Chronic exposure to glufosinate-ammonium induces spatial memory impairments, hippocampal MRI modifications and glutamine synthetase activation in mice

André-Guilhem Calas, Olivier Richard, Sandra Même, Jean-Claude Beloeil, Bich-Thuy Doan, Thierry Gefflaut, William Même, Wim E Crusio, Jacques Pichon, Céline Montecot

Chronic exposure to glufosinate-ammonium induces spatial memory impairments, hippocampal MRI modifications and glutamine synthetase activation in mice

André-Guilhem Calas [a], Olivier Richard [a], Sandra Même [b], Jean-Claude Beloeil [b], Bich-Thuy Doan [b], Thierry Gefflaut [c], William Même [a], Wim E. Crusio [d], Jacques Pichon [a,*], Céline Montécot [a]

[a] Laboratoire de Neurobiologie, UPRES EA 2633, Université d'Orléans, Orléans, France
[b] Centre de Biophysique Moléculaire, CNRS UPR4301, Orléans, France
[c] SEESIB, Université Blaise Pascal, CNRS UMR6504, Clermont-Ferrand, France
[d] Centre de Neurosciences Intégratives et Cognitives, Université de Bordeaux, CNRS UMR5228, Talence, France

ABSTRACT

Glufosinate-ammonium (GLA), the active compound of a worldwide-used herbicide, acts by inhibiting the plant glutamine synthetase (GS) leading to a lethal accumulation of ammonia. GS plays a pivotal role in the mammalian brain where it allows neurotransmitter glutamate recycling within astroglia. Clinical studies report that an acute GLA ingestion induces convulsions and memory impairment in humans. Toxicological studies performed at doses used for herbicidal activity showed that GLA is probably harmless at short or medium range periods. However, effects of low doses of GLA on chronically exposed subjects are not known. In our study, C57BL/6J mice were treated during 10 weeks three times a week with 2.5, 5 and 10 mg/kg of GLA. Effects of this chronic treatment were assessed at behavioral, structural and metabolic levels by using tests of spatial memory, locomotor activity and anxiety, hippocampal magnetic resonance imaging (MRI) texture analysis, and hippocampal GS activity assay, respectively. Chronic GLA treatments have effects neither on anxiety nor on locomotor activity of mice but at 5 and 10 mg/kg induce (1) mild memory impairments, (2) a modification of hippocampal texture and (3) a significant increase in hippocampal GS activity. It is suggested that these modifications may be causally linked one to another. Since glutamate is the main neurotransmitter in hippocampus where it plays a crucial role in spatial memory, hippocampal MRI texture and spatial memory alterations might be the consequences of hippocampal glutamate homeostasis modification revealed by increased GS activity in hippocampus. The present study provides the first data that show cerebral alterations after chronic exposure to GLA.

1. Introduction

Exposure to pesticides is a standing occupational hazard for farm workers making them a high-risk group in terms of public health. Epidemiological studies suggest that neuropsychological deficits follow long-term exposure to pesticides (Baldi et al., 2001), especially organophosphates (Farahat et al., 2003; Fiedler et al., 1997; Roldan-Tapia et al., 2006). It has been shown that workers who perform long-term farm work display cognitive and neurobehavioral impairments (Kamel et al., 2003). The disturbances induced by long-term exposure to organophosphorous compounds and more particularly to organophosphates mainly affect speed of processing, visuo-motor performances, visuo-perceptual abilities, anxiety, verbal abstraction, attention, and memory (Farahat et al., 2003; Roldan-Tapia et al., 2006). Different behavioral endpoints are used as indicators of neurotoxicity by the U.S. Food and Drug Administration: problems of motor coordination, sensory deficits, learning and memory dysfunctions, and altered states (Sobotka et al., 1996).

Phosphinothricin is an amino acid that is structurally related to glutamate and belongs to the organophosphorous family. Its ammonium salt, glufosinate-ammonium (GLA) is the active compound of worldwide-used broad-spectrum herbicides (such as Basta®). The relatively small amounts of GLA currently used in agriculture can be expected to increase in the near future, since

* Corresponding author. Tel.: +33 2 3849 4919; fax: +33 2 3841 7244.
E-mail address: jacques.pichon@univ-orleans.fr (J. Pichon).

genetically modified organisms (like soybean, cotton, maize, sugar beet and canola) resistant to this herbicide are being introduced on a commercial scale (D'Halluin et al., 1992). Indeed, such a resistance allows farmers to use GLA-containing herbicides in order to improve their crop yield by eliminating undesirable weeds with doses tolerated by GLA-resistant crops. Moreover, appearance of weeds naturally resistant to the glyphosate (Behrens et al., 2007), the currently most used herbicide in the world (Roundup®), would confer to GLA-containing compounds the role of substitute herbicide (Service, 2007).

GLA acts as a competitive and irreversible inhibitor of glutamine synthetase (GS, EC 6.3.1.2) in plants. This enzyme allows the plant to fix nitrogen from the soil as ammonium ions. GLA treatment leads to reduced glutamine amounts and increased ammonia levels in the plant's tissues (Lea et al., 1984). This causes photosynthesis to stop and as a result, the plant dies within a few days. GS is also present in almost every tissue in all animal species investigated so far (Meister, 1974). In the vertebrate nervous system, GS is present throughout the brain where it plays a central role in the metabolic regulation of glutamate, the major excitatory brain neurotransmitter (Bak et al., 2006). GS is exclusively localized in glial cells where it enables transformation of glutamate into glutamine by fixation of ammonia. We have previously shown that GLA also inhibits mammalian GS *in vitro* (Lapouble et al., 2002). Precedent studies have already established GLA toxicity on mouse embryos development in 48 h cultures with significant embryolethality and major morphological head defects (Watanabe and Iwase, 1996) and more specifically apoptosis in the neuroepithelium (Watanabe, 1997). Furthermore, direct exposure of rat cerebellum to acute doses of GLA by use of a microdialysis system causes alteration of glutamate neurotransmission (Nakaki et al., 2000). We have also shown that acute administration of a single high dose (75 mg/kg i.p.) of GLA induced in mice tonic and generalised seizures that we characterized by means of behavioral and electroencephalographic study (Lapouble et al., 2002).

Suicide attempts carried out by ingestion of high quantities of GLA-containing herbicide have been described in Japan. All the patients developed various neurological symptoms such as seizures and loss of memory (Hori et al., 2003; Ohtake et al., 2001; Tanaka et al., 1998; Watanabe and Sano, 1998). Acute effects of GLA exposure are well known through reports of these suicide attempts, whereas the effects of long-term exposure at low dose of GLA remain largely unknown and no specific epidemiological study has been carried out so far to study the effects of chronic exposure to this pesticide on the central nervous system. For this investigation we have therefore developed an experimental animal model of chronic GLA treatment in mice. The present study examines the effects of chronic GLA treatment at low and medium doses on spatial memory, anxiety, and motor activity by means of specific behavioral tasks. Because the hippocampus seems to be a brain structure sensitive to GLA, as reported in a magnetic resonance imaging (MRI) study of a recent case of human acute poisoning (Park et al., 2006), and because it plays a crucial role in spatial memory and anxiety in animals (Bannerman et al., 2004), MRI texture and glial GS activity were analyzed in the hippocampus in our model. MRI, a non-ionizing and non-invasive technology coupled to texture analysis (TA) is a powerful tool for our study because of its remarkable sensitivity and its high-resolution potency for identifying morphological and cellular alterations. MRI-based TA methods have already been described for cerebral studies in human suffering from brain diseases (Herlidou-Meme et al., 2003; Sankar et al., 2007; Yu et al., 2001) and in small animals (Yu et al., 2004).

2. Materials and methods

2.1. Animals and treatments

Male C57BL/6J mice (20–25 g) were obtained from the Centre de Distribution, Typage et Archivage animal (CDTA, UPS-44, CNRS, Orléans, France) at 8 weeks of age. The animals were housed for 2 weeks prior treatments under a 12-h light/dark cycle (light on at 7:30 a.m.) at constant temperature of 23 ± 1 °C with free access to food and water, except during the radial-maze test (see below). They were divided into four experimental groups of at least 13 animals each. GLA-treated groups were injected three times a week with a single dose of GLA dissolved in water containing 0.9% NaCl as vehicle (2.5, 5 or 10 mg/kg i.p.) during 12 weeks. Mice were weighed weekly before the first treatment of the week. The injection volume was 5 μL/g for all doses. Control animals received a comparable i.p. injection of vehicle. The experimental protocols were approved by the Regional Animal Care and Use Committee (CREEA Centre-Limousin, France, file number UNI45-001/10.15.2005).

2.2. Chemicals

GLA was obtained from Riedel-de Haën (Sigma–Aldrich; Isle d'Abeau, France) or was purified from commercially available herbicides: the solution (10 mL) containing 120 g/L of GLA was passed through a column of Dowex 50WX8 resin (H^+ form, 50 mL). The column was washed with 300 mL of water to elute the additives. A 1-M ammonia solution (100 mL) was then used to elute GLA. The ninhydrine positive fractions were combined, concentrated to dryness under reduced pressure and finally dried under vacuum (0.1 mmHg) for 48 h. GLA (1.25 g) was isolated as a white solid. Comparison with an analytical sample (Riedel-de Haën) by nuclear magnetic resonance spectroscopy confirmed the structure and the purity (>98%) of GLA.

2.3. Behavioral study

All along the 2 weeks that last the behavioral study, the mice were housed individually, in the same controlled environment and in the very room where the tests were to be performed. The light was adjusted at 28 ± 2 lux at each test stage. During the first week the mice were subjected to the radial-maze test. The second week of tests began with the elevated plus maze followed by the open-field test. In order to avoid potential acute effects, GLA treatments were carried out after a behavioral session. This allowed at least a delay of 15–18 h between GLA administration and the following session. All behavioral recordings were carried out with the experimenter blind to the treatment the mice had received. We used 28, 16, 13, and 22 animals, respectively for the vehicle, 2.5, 5, and 10 mg/kg treatments.

2.3.1. Radial-maze test

Spatial learning was tested in a radial-maze similar to the one described by Crusio and Schwegler (2005). The central part measured 25 cm in diameter. The eight arms (25-cm long, 6-cm high and 6-cm wide) were enclosed and made of transparent Plexiglas with a dark grey floor. Arm entrances could be blocked by lowering clear Plexiglas guillotine doors. At the end of each arm, some food pellets were deposited behind a perforated iron wall to prevent the mice from smelling the presence or absence of a food reward. Just in front of this perforated wall, a small food pellet (approximately 10 mg) was deposited behind a small black rod that prevented the animal from seeing the reward. The maze was always oriented in space in the same way. It was placed directly on the floor of the spatially richly structured mouse room in order to

avoid possible elevation-induced anxiety. In addition, five extra-maze cues were provided close (2–15 cm) to the arms in a fixed configuration.

On the first day, each mouse was subjected to a habituation session in which it was allowed to explore the maze freely for 20 min. Arm doors remained open, and no food was accessible in the maze. Mice were partially deprived of food, but not water during the 5 days of the test. They reached about 85% of their initial body weight within 24 h and were maintained at this level throughout the rest of the experiment. The habituation trial was followed 24 h later by one trial per day during 5 days, during which all 8 arms contained a food reward. Animals were confined between arm-visits for 5 s on the central platform by lowering the guillotine doors. This procedure has been shown to avoid the use of kinesthetic strategies (Schwegler et al., 1990). An error was noted if animals failed to eat the food reward or revisited an arm in which the food reward had been previously eaten. In addition, the number of different arms visited among the first eight arms sampled was recorded as new entries. The number of arms entered per minute was taken as an index of locomotor activity. Trials ended when animals had found and eaten all eight rewards. Subsequently, they were weighed, returned to their home cages, and administered an appropriate amount of food to maintain them at 85% of free-feeding body weight. The maze was wiped, but not rinsed between different subjects. The test took place 10 weeks after the beginning of chronic GLA treatment.

2.3.2. Elevated plus maze (EPM) test

The EPM consisted of a black Plexiglas 4 armed platform with all arms arranged in the shape of a plus and raised 46.5 cm above the floor. All arms were 5.5 cm in width and 31 cm in length and joined in the center to a 30.25-cm^2 central platform. Two arms facing each other were protected (enclosed). The two remaining arms facing each other and arranged perpendicular to the protected arms remained open. Protected arms were surrounded by 16 cm high transparent Plexiglas walls, which were open at the top, while open arms were bounded by a 0.6-cm high edge providing additional grip for the animals.

At the start of each test, mice were placed in the center square facing the same open arm and were allowed to move freely. The mice were evaluated in a single 5-min session. After each test, the maze was cleaned in order to remove any residue or odors. A mouse was considered to have entered an arm when all four paws were on the arm. The standard measure of anxiety was the ratio of the time spent in the open arms of the maze divided by the total time spent in any arm of the maze. Smaller ratios indicate less open arm exploration or higher levels of anxiety (Lister, 1987).

2.3.3. Open-field test

The open-field was used to evaluate the exploratory activity of the animal. The open-field area was made of Plexiglas (grey walls and white floor, 50 cm × 50 cm × 29 cm) divided into nine squares (16.6 cm × 16.6 cm) with blue adhesive tape. Each animal was placed singly in the middle of the field and its behavior recorded in a single 5-min session. Following each open-field test, fecal boli were removed and the box was cleaned with a humid sponge. The observed parameter was total crossings which corresponded to the number of lines crossed with the four paws (locomotor activity).

Subsequently to the behavioral study, five animals from each group under treatment were picked randomly and submitted to MRI exams. Treatments were performed at least 15 h prior to MRI exams to avoid potential acute GLA effects.

2.4. MRI study

2.4.1. MR images acquisition

MR experiments were obtained with a 9.4-T horizontal magnet (94/21 USR Bruker Biospec, Wissembourg, France), equipped with a 950-mT/m gradient set. The mouse head was placed with a stereotaxic system made of a tooth bar and two ear bars within a linear homogeneous shielded coil (inner diameter 35 mm). The body temperature was maintained constant during the experiments with a heating water bed. The animals were put under gaseous anaesthesia during MRI exams (50% N_2O: 0.7 L/min; 50% O_2: 0.7 L/min; Isoflurane 1.5%, TEM, France). The breathing rate was measured during the acquisitions to monitor the anaesthetic output. A first series of sagittal images was performed with an eight echoes-RARE sequence (FOV = 1.5 cm × 1.5 cm, matrix size = 256 × 256, slice thickness = 1 mm, TE = 46 ms, TR = 5 s) leading to an in plane spatial resolution of 59 μm × 59 μm. Then, 15 axial images were acquired with the same sequence but with a slice thickness of 0.5 mm. The total duration of the MR acquisitions was 50 min. The central slice of the 15 axial images package always corresponded to bregma −0.94 mm so that the slices were located at the same positions for all mice.

2.4.2. MRI TA

TA covers several methods enabling quantification and statistical analysis of the grey level values on an image region of interest (ROI) (Haralick, 1979). A complete mathematical description of the TA parameters has already been published (Herlidou-Meme et al., 2003; Herlidou et al., 1999, 2004). Post-acquired images (see above) were transferred to an external computer for computational analysis. A ROI was manually delineated in the hippocampus as big as possible in both hemispheres on five axial slices for each dose (vehicle, GLA 2.5, 5, and 10 mg/kg). These ROI were analyzed with four different TA methods (histogram, calculations of the co-occurrence, run-length, and gradient matrices) using a custom-made software. Each ROI was then characterized with its own texture profile (formed with the different texture parameters calculated before). Correspondence factorial analyses (CFA) were performed to discriminate between all the profiles (vehicle, GLA 2.5, 5, and 10 mg/kg) using XIstat software (XIstat7.5©1995–2004, Addinsoft). This data analysis method also allows keeping the most discriminating texture parameters.

After completion of the MRI exams, all mice were gathered and randomly split into two groups. One group was used for histological study, whereas the other was submitted to GS activity assays.

2.5. Histological study

Animals were deeply anaesthetized with isoflurane and perfused through the left ventricule with a cold saline solution containing 50 IU/mL of heparin followed by a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Fixed brains were removed and kept in the same cold fixative solution during 16 h at 4 °C and then cryoprotected with 30% (w/v) sucrose in 0.1 M phosphate buffer (pH 7.4) during 24 h at 4 °C. Brains were frozen in isopentane at −50 °C during 1 min. Coronal 20-μm thick brain sections were cut on a cryomicrotome (Leica CM3050S) −1.94 mm from Bregma according to the mouse brain atlas edited by Paxinos and Franklin (2001). The sections were mounted on gelatin-coated slides and stained with cresyl violet. Only vehicle and 10 mg/kg brains were examined by conventional microscopy at 100× magnification. The observer, blind to the treatment, evaluated the severity of the neuronal damage on the hippocampal sections of each brain. The damage was defined on the basis of the following

morphological characteristics: pycnotic nuclei, shrunken perikarya, microvacuolation, absence of cellular bodies, and presence of cellular debris in CA1, CA2, CA3, CA4 pyramidal cell layers and dentate gyrus granule cells layer of the hippocampus.

2.6. GS activity assay

Photometric determination of GS activity is based on the formation of a L-γ-glutamylhydroxamate ferric chloride complex and was performed according to Wellner and Meister (1966) with slight modifications. Samples of hippocampus were removed on ice from each animal brain. Tissues were frozen using liquid nitrogen and then kept at −20 °C until use. Samples were sonicated in a 150/5-mM KCl/cysteine solution. Homogenates were incubated for 10 min at 37 °C, centrifuged 30 000 × *g* for 1 h 30 at 4 °C and supernatants were used to dose GS activity. The reactive mixture consisted of 100 mM imidazole pH 7.2, 50 mM sodium L-glutamate, 10 mM β-mercaptoethanol, 20 mM sodium ATP, 40 mM $MgCl_2$ and 100 mM hydroxylamine pH 7.2. The reaction was initiated by the addition of 75 μL of the reactive mixture to 50 μL of the supernatant of each sample. The reaction was quenched after 1 h at 37 °C with 150 μL of 0.37 M $FeCl_3$/0.67 M HCl/0.2 M trichloroacetic acid. This mixture was then incubated for 30 min at 4 °C before absorbance was read at 530 nm. All absorbances were within the linear range of the γ-glutamylhydroxamate standard curve. Protein concentration was determined with a dye-binding assay (DC Protein Assay, BioRad) using bovine serum albumin as a standard. For each animal, determinations were performed in triplicate. GS activity was expressed in mM of γ-glutamylhydroxamate formed per hour per milligram of proteins at 37 °C.

2.7. Statistical analysis

Death during treatments was analyzed by χ^2-test to determine if it was to be imputed to GLA treatment. For the body weight time course a repeated-measures analyses of variance (ANOVA) over the first 10 weeks of treatment was used. The results of the GS assay and the behavioral tests except for the radial-maze test were evaluated by one-way ANOVA followed by Scheffé *post hoc* tests, with treatment as between-subjects factor. For the radial-maze test a repeated-measures ANOVA with days as additional within-subjects factor was used, whereas treatment comparisons for individual days were done by means of one-way ANOVAs. As some animals did not always take the food reward the first time they entered an arm of the radial-maze on days 1 and 2, only the data from days 3–5 were used for statistical evaluation (Schwegler et al., 1990). Student's *t*-tests were used to test whether numbers of new entries deviated from 5.3, the expected value for a random choice of an arm (Olton and Schlosberg, 1978). All values are expressed as means ± standard error of the mean (S.E.M.).

3. Results

3.1. Chemicals

No differences were observed in the GS assay and the behavioral study between the treatments at the dose of 5 mg/kg performed with either commercial or purified GLA (data not shown). In what follows, the results obtained with commercial and purified GLA were pooled for this dose.

3.2. Behavioral study

A repeated-measures ANOVA over the first 10-week treatment testified that the increases in body weight did not differ between the four treated groups ($F_{3,75} = 1.17$, $p = 0.3250$, Fig. 1). A 12% death rate was observed in the 10 mg/kg GLA-treated group, whereas no death was to be reported in the other groups. Nevertheless, a χ^2-analysis showed that this rate was not significant.

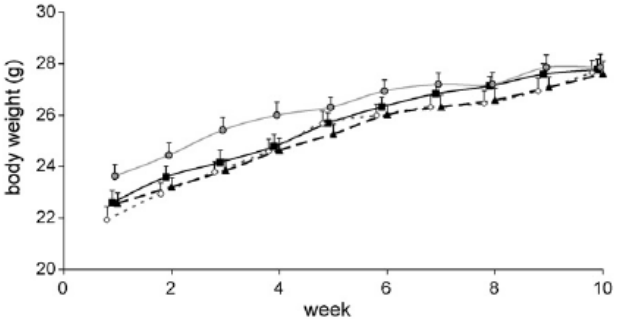

Fig. 1. Time course of mouse body weight during the 10-week treatment. For clarity, some dots were *x*-axis shifted in order not to stack S.E.M. lines. Results are expressed as means ± S.E.M. ($n = 28, 16, 13, 22$, respectively for the vehicle, GLA 2.5, 5, and 10 mg/kg groups). (—■—) Vehicle; (···◇···) GLA 2.5 mg/kg; (– ▲ –) GLA 5 mg/kg; (—●—) GLA 10 mg/kg.

3.2.1. Radial-maze test

Both the vehicle-treated and the GLA-treated mice showed a gradual increase in the number of new entries (Fig. 2A) and a decrease in the number of errors (Fig. 2B) with advancing training. Mean new entries were significantly higher than 5.3 in the vehicle and 2.5 mg/kg GLA-treated animals ($p < 0.01$ and $p < 0.001$, respectively, on the fifth day of training), but not in the 5 and 10 mg/kg GLA groups (both $p > 0.05$ on the fifth day), indicating a random selection of arms for 5 and 10 mg/kg GLA-treated mice but not for vehicle and 2.5 mg/kg GLA-treated mice. A repeated-measures ANOVA over the last three days of training indicated a tendency towards significance for the total number of errors made ($F_{3,75} = 2.45$, $p = 0.070$) as well as a borderline significant effect for the numbers of new entries ($F_{3,75} = 2.68$, $p = 0.053$), whereas the activity index (Fig. 2C) did not differ between treatment groups ($F_{3,75} = 0.79$, $p > 0.50$). However, on none of the last three days of training did the numbers of new entries ($F_{3,75} = 1.917$, $p = 0.1344$; $F_{3,75} = 1.1$, $p = 0.3547$; $F_{3,75} = 1.578$, $p = 0.202$), total errors ($F_{3,75} = 1.913$, $p = 0.1351$; $F_{3,75} = 2.098$, $p = 0.1079$; $F_{3,75} = 1.054$, $p = 0.3741$), and the activity index ($F_{3,75} = 0.393$, $p = 0.7586$; $F_{3,75} = 0.593$, $p = 0.6218$; $F_{3,75} = 1.254$, $p = 0.2966$) (Fig. 2A–C, respectively) differ significantly between the vehicle-treated and the GLA-treated mice.

3.2.2. Elevated plus maze test

There were no statistically significant differences between GLA-treated animals and controls for percentage open arm time (Fig. 3; $F_{3,75} = 1.635$, $p = 0.1885$).

3.2.3. Open-field test

As shown in Fig. 4, GLA did not change the exploratory locomotor activity of C57BL/6J mice in the open-field test at any dose ($F_{3,75} = 2.030$, $p = 0.1169$).

3.3. MRI TA

The various CFAs made it possible to eliminate the redundant texture parameters and to keep only the most relevant ones for discrimination between the different ROIs with $p < 0.05$ used as threshold for statistical significance. Fifty-one texture parameters were available after computing the various TA methods, but only

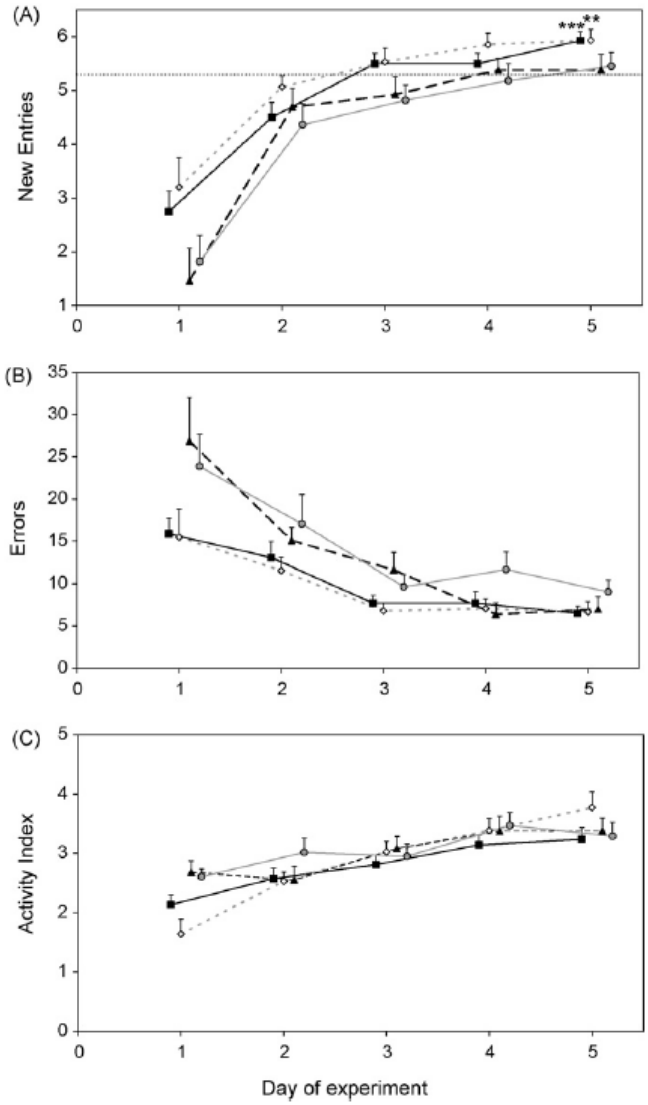

Fig. 2. Results obtained in the radial-maze test. (A) Number of new entries during the first eight choices. A horizontal dash line shows the theoretical value of 5.3 expected if animals choose arms randomly. (B) Number of total errors. (C) The activity index (number of arms entered per minute). For clarity, some dots were *x*-axis shifted in order not to stack S.E.M. lines. Results are expressed as means ± S.E.M. (n = 28, 16, 13, 22, respectively for the vehicle, GLA 2.5, 5, and 10 mg/kg groups). $^{**}p < 0.01$; $^{***}p < 0.001$ vs. 5.3. (—■—) Vehicle; (···◇···) GLA 2.5 mg/kg; (– ▲ –) GLA 5 mg/kg; (—●—) GLA 10 mg/kg.

four of them were kept: run-length distribution (from run-length matrix), contrast, sum average and sum variance (from the co-occurrence matrix). The results of the CFA performed to discriminate between the different texture profiles corresponding to the different doses are shown in Fig. 5. All texture profiles can be classified in two parts with no misclassification: part A for vehicle and 2.5 mg/kg GLA-treated mice and part B for 5 and 10 mg/kg GLA-treated mice. The in-plane representation is given with the two first factorial axes, which contain 96% of the whole data set (70% on factorial axis 1 and 26% on factorial axis 2). Factorial axes 1 and 2 are weighted by contrast (40.5% for axis 1 and 3.3% for axis 2, respectively), sum average (6.4 and 67.9%), sum variance (19.2 and 0.1%) and run-length distribution (33.9 and 23.7%).

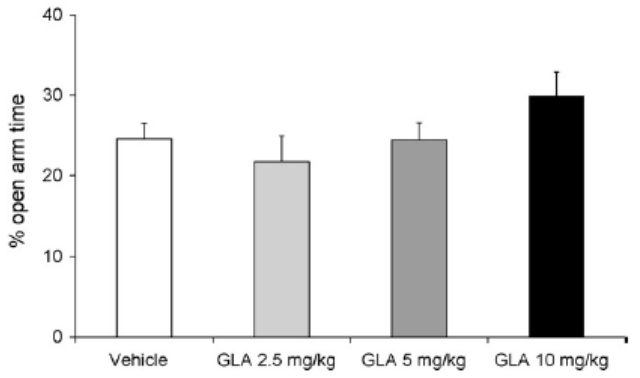

Fig. 3. Effect of GLA in the elevated plus-maze test in C57BL/6J male mice on percentage of time spent in open arms with respect to total time spent in the arms. Results are expressed as means ± S.E.M. (n = 28, 16, 13, 22, respectively for the vehicle, GLA 2.5, 5, and 10 mg/kg groups).

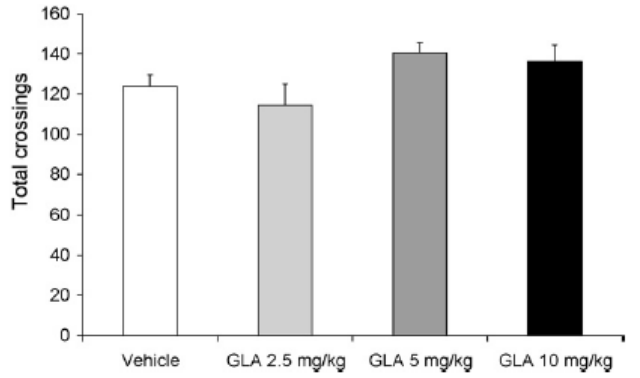

Fig. 4. Effect of GLA on locomotor activity measured as total line crossings during 5 min in the open-field test in C57BL/6J mice. Results are expressed as means ± S.E.M. (n = 28, 16, 13, 22, respectively for the vehicle, GLA 2.5, 5, and 10 mg/kg groups).

3.4. Histological study

Observation of the CA1, CA2, CA3 and CA4 pyramidal cells layers and dentate gyrus granular cells layer following cresyl violet coloration did not reveal any neuronal death in the hippocampus after chronic exposure to GLA at a dose of 10 mg/kg. Indeed, the observer did not notice any differences between the sections from vehicle and from mice submitted to 10 mg/kg of GLA (Fig. 6).

3.5. GS activity assay

In vehicle-treated mice, the mean value of *in vitro* hippocampal GS activity was 0.277 ± 0.02 mM γ-glutamylhydroxamate formed/h/mg protein. GS activity increase in the 2.5 mg/kg GLA-treated group was not significant compared to the GS activity in vehicle-treated mice. In 5 and 10 mg/kg GLA-treated mice, the mean value increased significantly by 169 and 146%, respectively ($F_{3,37}$ = 4.738, p = 0.0068, both $p < 0.05$ with Scheffé *post hoc* test (Fig. 7)).

4. Discussion

The major finding of the present study is that GLA, an irreversible inhibitor of GS in plants used as an herbicide, induced metabolic and texture modifications in the hippocampus as well as mild learning impairments in C57BL/6J mice after 10 weeks of treatment with 3 weekly i.p. injections of 5 and 10 mg/kg.

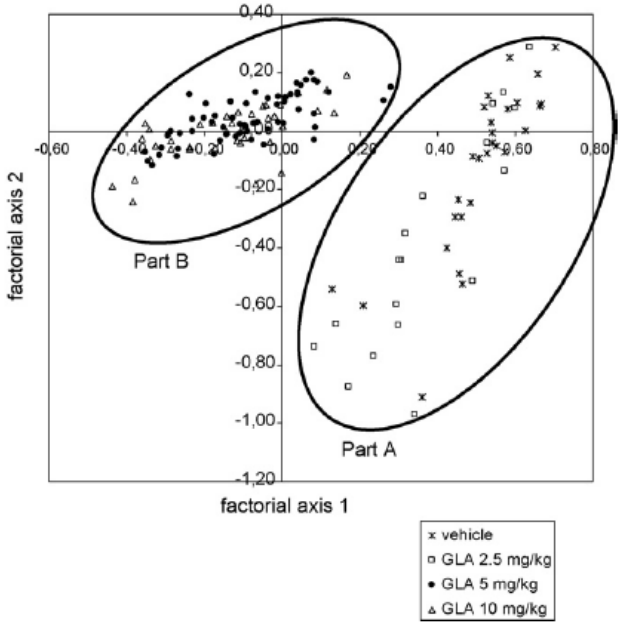

Fig. 5. Correspondence factorial analyses of the hippocampus ROI for vehicle and GLA doses 2.5, 5 and 10 mg/kg. The graph can be divided into two parts with no misclassified profiles. Part A consists of texture profiles for vehicle and dose 2.5 mg/kg; part B corresponds to the texture profiles for doses 5 and 10 mg/kg.

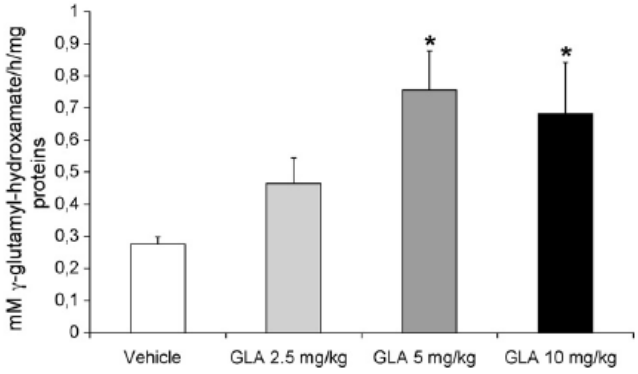

Fig. 7. Hippocampal glutamine synthetase activity expressed as mM γ-glutamyl-hydroxamate formed per hour per milligram proteins at 37 °C. Results are expressed as means ± S.E.M. (n = 16, 7, 6, and 12, respectively for the vehicle, GLA 2.5, 5, and 10 mg/kg groups.).*$p < 0.05$ vs. control.

Ubiquitous in the brain, GS is located mainly in astrocytes where it plays a pivotal role in glutamate homeostasis but also in ammonia detoxification via an amidation reaction of glutamate. Because of the known inhibition of cerebral GS *in vitro* by GLA (Lapouble et al., 2002), its effects on hippocampal GS activity were investigated here. Surprisingly, chronic GLA treatments do not inhibit hippocampal GS but, on the contrary, drastically increase GS activity in the 5 and 10 mg/kg GLA dose groups. Our results seemed to be in contradiction with a previous study performed on Wistar rats, where animals were fed a diet containing 0, 40, 200, 1000 and 5000 ppm GLA for 28 days. Whole brain GS activity only decreased in the 5000 ppm GLA treatment (corresponding to a 530 mg/kg/day treatment) by 42% and also showed a significant diminution of brain glutamine (Hack et al., 1994). The difference with our results could be explained by procedural differences in length (two times shorter), intensity (100-fold higher), or nature (oral vs. i.p.) of the GLA treatments and/or species (rat vs. mice) differences in sensitivity. Indeed, rats could react differently to xenobiotics exposure compared to other species and mice in particular.

GLA could enhance GS activity in mice brain in several, non-exclusive, ways, by increasing the (i) specific activity of the enzyme; (ii) *de novo* synthesis of the protein; (iii) number of GS expressing cells. GLA is able to bind (Robinson et al., 1985) and to activate, directly or indirectly (Lapouble et al., 2002; Matsumura et al., 2001; Nakaki et al., 2000), glutamate-*N*-methyl-D-aspartate (NMDA) receptors. It is known that activation of these receptors plays a crucial role in the modulation of GS activity (Davenport Jones et al., 1998; Kosenko et al., 1994). The ammonium part of the drug could produce hyperammonemia that might directly stimulate GS activity (for review see Suarez et al. (2002)). An excess of ammonia may also lead to an altered glutamate binding to NMDA receptors (Peterson et al., 1990). Whatever the origin, the striking heightener in hippocampal GS activity after GLA treatment might affect the glutamate neurotransmitter system and could perturb the physiological integrity of this brain area, strongly involved in the memory.

The data obtained with the radial-maze test show that GLA 5 and 10 mg/kg lead to a deficit in spatial learning as the number of new entries of mice subjected to these treatments did not significantly exceed the value of 5.3 that is expected if animals choose arms randomly, on any of the 5 days of testing. However, the absence of significant differences with the control group indicates that this deficit is moderate. Memory impairment in the radial-maze task was also shown in adult rats that were prenatally exposed to chlorpyrifos, one of the most widely used organopho-

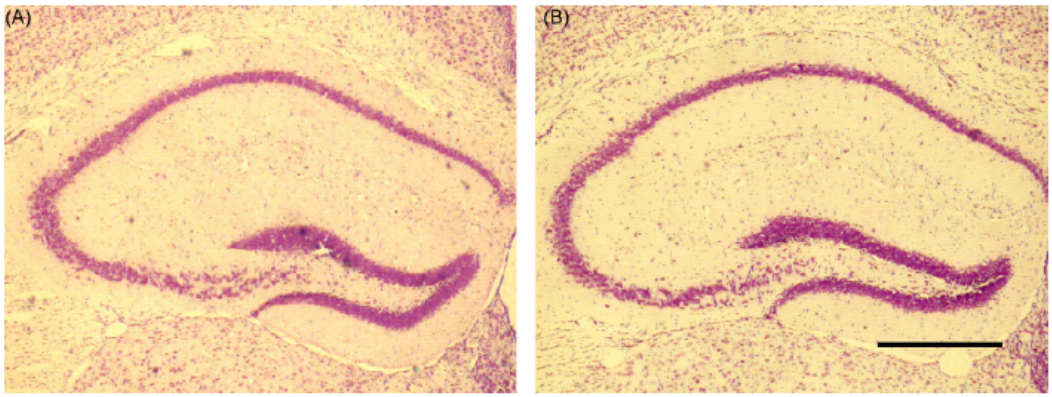

Fig. 6. Representative micrographs of hippocampal sections stained with cresyl violet of a vehicle-treated mice (A) and a 10 mg/kg GLA-treated mice (B). Scale bar = 500 μm.

sphate insecticides (Icenogle et al., 2004; Levin et al., 2002). The impairment of spatial orientation in the radial-maze of the 5 and 10 mg/kg treated animals might be due to hippocampal perturbations. The hippocampus plays a critical role in memory for the place and the acquisition of the radial arm maze (a non-aversive food-motivated spatial orientation test) depends on an intact hippocampal system (Martin and Clark, 2007). The nature of the neuronal consequences of human poisoning with high doses of GLA supports this hypothesis: accidental ingestion of GLA induces anterograde amnesia with concurrent bilateral hippocampal lesions observed by MRI (Park et al., 2006). Furthermore, the hippocampus contains the highest density of NMDA receptors in the brain (Monaghan and Cotman, 1985), where they play a major role in spatial learning and memory (for review, see Nakazawa et al. (2004)). It is therefore conceivable that chronic exposure to GLA could affect hippocampal memory processes through impairments of NMDA receptor activation and/or disturbance of the glutamate–glutamine cycle via GS activation in the hippocampus. Nevertheless, memory appears to be relatively insensitive to perturbation of GS activity since it has been demonstrated that an 80% GS inhibition by chronic treatment with methionine sulfoximine, a selective GS inhibitor, does not influence hippocampus-dependent spatial learning and memory in C57BL/6J mice (Blin et al., 2002). Thus, it seems more probable that chronically GLA-treated mice develop hippocampal memory impairments through disrupted activation of NMDA receptors.

GLA treatment does not seem to induce an increase of locomotor activity in the open-field. However, Li et al. (1999) have shown that, after hippocampal lesions, deficits of hippocampus-dependent spatial memory are accompanied by locomotor impairments. Hippocampal lesions are known to induce memory deficits or an increase of locomotor activity which both appear to be closely correlated with the extent of neuron loss in the hippocampal CA3 subfield (Andersen et al., 1997; Gilbert and Kesner, 2006). Here we show that evaluation of the damage in the pyramidal cell layer of Ammon's horn after 10 mg/kg GLA treatment did not induce any neuronal death in the hippocampus (Fig. 6). The lack of effect on locomotion in our study could also be linked to the fact that our data show only moderate spatial memory deficits and a lack of neuronal damage compared to their results (Li et al., 1999).

GLA treatment does not seem to induce an effect on anxiety as shown by our EPM results. Anxiety is known to be regulated by hippocampus (Bannerman et al., 2004; Li et al., 1999) but recent study suggest that, within the hippocampus, anxiety and memory are regionally dissociated (Bertoglio et al., 2006) and, more precisely, ventral hippocampus seems to be involved in the regulation of defensive behaviors related to anxiety whereas the dorsal hippocampus has a preferential role in spatial learning (Bannerman et al., 2004). A more region-specific MRI texture study needs to be carried out in order to discriminate GLA treatment effects on dorsal and ventral hippocampus.

The results obtained with the MRI TA of the hippocampus show that two distinct texture profiles exist, (i) vehicle and 2.5 mg/kg GLA and (ii) 5 and 10 mg/kg GLA, suggesting that a dose between 2.5 and 5 mg/kg could be considered as a limit to detect these small changes with TA. Such a result confirms that the hippocampus is a target of chronically administered GLA in mice. Structural modifications in the hippocampus revealed by TA following GLA treatment might result from metabolic changes in neurons or astrocytes or in both of them. The increased glial GS activity we showed may lead to an accumulation of glutamine in astrocytes. Such potential accumulation would produce osmotic stress which might induce astrocytes swelling (De Keyser et al., 2008). MRI TA based on proton resonance mainly assesses the water distribution in the tissue. With this technique, even a subtle change in the amount of water in different parts of the tissue could be established.

Our study strongly suggests that MRI TA, a non-invasive technology, is able to detect minute structural changes that are not visible with classical histological techniques. Still these tiny structural modifications could reflect moderate alterations of the physiology of glial cell which may lead to slight memory impairments. TA-associated MRI technique is a powerful tool that could be used to assess and to follow any human chronic poisoning which affects cerebral function.

The present data show structural modifications in the hippocampus, as revealed by MRI TA, and modification of glutamate metabolism, as suggested by increased GS activity, which may both lead to mild memory impairments after a chronic treatment with three weekly injections of 5 and 10 mg/kg GLA for 10 weeks.

In conclusion, the present study provides the first data that indicate cerebral modifications consecutively to a chronic exposure to GLA, the active ingredient of some commercial herbicides.

Acknowledgements

This work was made possible thanks to financial supports from the French National Research Agency (ANR, grant HERBITOX #2006 SEST 18 01) and the French BIOSIMS network (FNADT and Région Centre). The fellowship allocated to A.G. C. by the Région Centre is gratefully acknowledged. We thank J. Moreno and Ph. Moreau for efficient and careful housing of the mice. We also thank T. Angevin for his constructive comments.

References

Andersen MB, Zimmer J, Sams-Dodd F. Postischemic hyperactivity in the Mongolian gerbil correlates with loss of hippocampal neurons. Behav Neurosci 1997;111(6):1205–16.

Bak LK, Schousboe A, Waagepetersen HS. The glutamate/GABA–glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer. J Neurochem 2006;98(3):641–53.

Baldi I, Filleul L, Mohammed-Brahim B, Fabrigoule C, Dartigues JF, Schwall S, et al. Neuropsychologic effects of long-term exposure to pesticides: results from the French Phytoner Study. Environ Health Perspect 2001;109(8):839–44.

Bannerman DM, Rawlins JN, McHugh SB, Deacon RM, Yee BK, Bast T, et al. Regional dissociations within the hippocampus-memory and anxiety. Neurosci Biobehav Rev 2004;28(3):273–83.

Behrens MR, Mutlu N, Chakraborty S, Dumitru R, Jiang WZ, Lavallee BJ, et al. Dicamba resistance: enlarging and preserving biotechnology-based weed management strategies. Science 2007;316(5828):1185–8.

Bertoglio LJ, Joca SR, Guimaraes FS. Further evidence that anxiety and memory are regionally dissociated within the hippocampus. Behav Brain Res 2006;175(1):183–8.

Blin M, Crusio WE, Hevor T, Cloix JF. Chronic inhibition of glutamine synthetase is not associated with impairment of learning and memory in mice. Brain Res Bull 2002;57(1):11–5.

Crusio WE, Schwegler H. Learning spatial orientation tasks in the radial-maze and structural variation in the hippocampus in inbred mice. Behav Brain Funct 2005;1(1):3.

D'Halluin K, De Block M, Denecke J, Janssens J, Leemans J, Reynaerts A, et al. The bar gene as selectable and screenable marker in plant engineering. Methods Enzymol 1992;216:415–26.

Davenport Jones JE, Fox RM, Atterwill CK. NMDA-induced increases in rat brain glutamine synthetase but not glial fibrillary acidic protein are mediated by free radicals. Neurosci Lett 1998;247(1):37–40.

De Keyser J, Mostert JP, Koch MW. Dysfunctional astrocytes as key players in the pathogenesis of central nervous system disorders. J Neurol Sci 2008;267(1–2):3–16.

Farahat TM, Abdelrasoul GM, Amr MM, Shebl MM, Farahat FM, Anger WK. Neurobehavioural effects among workers occupationally exposed to organophosphorous pesticides. Occup Environ Med 2003;60(4):279–86.

Fiedler N, Kipen H, Kelly-McNeil K, Fenske R. Long-term use of organophosphates and neuropsychological performance. Am J Ind Med 1997;32(5):487–96.

Gilbert PE, Kesner RP. The role of the dorsal CA3 hippocampal subregion in spatial working memory and pattern separation. Behav Brain Res 2006;169(1):142–9.

Hack R, Ebert E, Ehling G, Leist KH. Glufosinate ammonium—some aspects of its mode of action in mammals. Food Chem Toxicol 1994;32(5):461–70.

Haralick RM. Statistical and structural approaches to texture. Proc IEEE 1979;67:786–804.
Herlidou-Meme S, Constans JM, Carsin B, Olivie D, Eliat PA, Nadal-Desbarats L, et al. MRI texture analysis on texture test objects normal brain and intracranial tumors. Magn Reson Imaging 2003;21(9):989–93.
Herlidou S, Grebe R, Grados F, Leuyer N, Fardellone P, Meyer ME. Influence of age and osteoporosis on calcaneus trabecular bone structure: a preliminary in vivo MRI study by quantitative texture analysis. Magn Reson Imaging 2004;22(2):237–43.
Herlidou S, Rolland Y, Bansard JY, Le Rumeur E, de Certaines JD. Comparison of automated and visual texture analysis in MRI: characterization of normal and diseased skeletal muscle. Magn Reson Imaging 1999;17(9):1393–7.
Hori Y, Tanaka T, Fujisawa M, Shimada K. Toxicokinetics of DL-glufosinate enantiomer in human BASTA poisoning. Biol Pharm Bull 2003;26(4):540–3.
Icenogle LM, Christopher NC, Blackwelder WP, Caldwell DP, Qiao D, Seidler FJ, et al. Behavioral alterations in adolescent and adult rats caused by a brief subtoxic exposure to chlorpyrifos during neurulation. Neurotoxicol Teratol 2004;26(1):95–101.
Kamel F, Rowland AS, Park LP, Anger WK, Baird DD, Gladen BC, et al. Neurobehavioral performance and work experience in Florida farmworkers. Environ Health Perspect 2003;111(14):1765–72.
Kosenko E, Kaminsky Y, Grau E, Minana MD, Marcaida G, Grisolia S, et al. Brain ATP depletion induced by acute ammonia intoxication in rats is mediated by activation of the NMDA receptor and Na+, K(+)-ATPase. J Neurochem 1994;63(6):2172–8.
Lapouble E, Montecot C, Sevestre A, Pichon J. Phosphinothricin induces epileptic activity via nitric oxide production through NMDA receptor activation in adult mice. Brain Res 2002;957(1):46–52.
Lea PJ, Joy KW, Ramos JL, Guerrero MG. The action of 2-amino-4-(methylphosphinyl)-butanoic acid (phosphinothricin) and its 2-oxo-derivative on the metabolism of cyanobacteria and higher plants. Phytochemistry 1984;23(1):1–6.
Levin ED, Addy N, Baruah A, Elias A, Christopher NC, Seidler FJ, et al. Prenatal chlorpyrifos exposure in rats causes persistent behavioral alterations. Neurotoxicol Teratol 2002;24(6):733–41.
Li H, Matsumoto K, Watanabe H. Different effects of unilateral and bilateral hippocampal lesions in rats on the performance of radial maze and odor-paired associate tasks. Brain Res Bull 1999;48(1):113–9.
Lister RG. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berlin) 1987;92(2):180–5.
Martin SJ, Clark RE. The rodent hippocampus and spatial memory: from synapses to systems. Cell Mol Life Sci 2007;64(4):401–31.
Matsumura N, Takeuchi C, Hishikawa K, Fujii T, Nakaki T. Glufosinate ammonium induces convulsion through *N*-methyl-D-aspartate receptors in mice. Neurosci Lett 2001;304(1–2):123–5.
Meister A. Glutamine synthetase of mammals. In: Boyer PD, editor. The enzymes. New York: Academic Press; 1974. p. 699–754.
Monaghan DT, Cotman CW. Distribution of *N*-methyl-D-aspartate-sensitive L-[3*H*]glutamate-binding sites in rat brain. J Neurosci 1985;5(11):2909–19.
Nakaki T, Mishima A, Suzuki E, Shintani F, Fujii T. Glufosinate ammonium stimulates nitric oxide production through *N*-methyl D-aspartate receptors in rat cerebellum. Neurosci Lett 2000;290(3):209–12.
Nakazawa K, McHugh TJ, Wilson MA, Tonegawa S. NMDA receptors, place cells and hippocampal spatial memory. Nat Rev Neurosci 2004;5(5):361–72.
Ohtake T, Yasuda H, Takahashi H, Goto T, Suzuki K, Yonemura K, et al. Decreased plasma and cerebrospinal fluid glutamine concentrations in a patient with bialaphos poisoning. Hum Exp Toxicol 2001;20(8):429–34.
Olton DS, Schlosberg P. Food-searching strategies in young rats: win-shift predominates over win-stay. J Comp Physiol Psychol 1978;92:609–18.
Park HY, Lee PH, Shin DH, Kim GW. Anterograde amnesia with hippocampal lesions following glufosinate intoxication. Neurology 2006;67(5):914–5.
Paxinos G, Franklin KBJ. The mouse brain in stereotaxic coordinates. 2nd ed. San Diego: Academic Press; 2001.
Peterson C, Giguere JF, Cotman CW, Butterworth RF. Selective loss of *N*-methyl-D-aspartate-sensitive L-[3*H*]glutamate binding sites in rat brain following portacaval anastomosis. J Neurochem 1990;55(2):386–90.
Robinson MB, Crooks SL, Johnson RL, Koerner JF. Displacement of DL-[3*H*]-2-amino-4-phosphonobutanoic acid ([3*H*]APB) binding with methyl-substituted APB analogues and glutamate agonists. Biochemistry 1985;24(10):2401–5.
Roldan-Tapia L, Nieto-Escamez FA, del Aguila EM, Laynez F, Parron T, Sanchez-Santed F. Neuropsychological sequelae from acute poisoning and long-term exposure to carbamate and organophosphate pesticides. Neurotoxicol Teratol 2006;28(6):694–703.
Sankar T, Bernasconi N, Kim H, Bernasconi A. Temporal lobe epilepsy: differential pattern of damage in temporopolar cortex and white matter. Hum Brain Mapp 2007.
Schwegler H, Crusio WE, Brust I. Hippocampal mossy fibers and radial-maze learning in the mouse: a correlation with spatial working memory but not with non-spatial reference memory. Neuroscience 1990;34(2):293–8.
Service RF. Glyphosate-the conservationist's friend? Science 2007;316(5828):1116–7.
Sobotka TJ, Ekelman KB, Slikker W Jr, Raffaele K, Hattan DG. Food and Drug Administration proposed guidelines for neurotoxicological testing of food chemicals. Neurotoxicology 1996;17(3–4):825–36.
Suarez I, Bodega G, Fernandez B. Glutamine synthetase in brain: effect of ammonia. Neurochem Int 2002;41(2–3):123–42.
Tanaka J, Yamashita M, Yamashita M, Matsuo H, Yamamoto T. Two cases of glufosinate poisoning with late onset convulsions. Vet Hum Toxicol 1998;40(4):219–22.
Watanabe T. Apoptosis induced by glufosinate ammonium in the neuroepithelium of developing mouse embryos in culture. Neurosci Lett 1997;222(1):17–20.
Watanabe T, Iwase T. Developmental and dysmorphogenic effects of glufosinate ammonium on mouse embryos in culture. Teratogen Carcinogen Mutagen 1996;16(6):287–99.
Watanabe T, Sano T. Neurological effects of glufosinate poisoning with a brief review. Hum Exp Toxicol 1998;17(1):35–9.
Wellner VP, Meister A. Binding of adenosine triphosphate and adenosine diphosphate by glutamine synthetase. Biochemistry 1966;5(3):872–9.
Yu O, Mauss Y, Namer IJ, Chambron J. Existence of contralateral abnormalities revealed by texture analysis in unilateral intractable hippocampal epilepsy. Magn Reson Imaging 2001;19(10):1305–10.
Yu O, Steibel J, Mauss Y, Guignard B, Eclancher B, Chambron J, et al. Remyelination assessment by MRI texture analysis in a cuprizone mouse model. Magn Reson Imaging 2004;22(8):1139–44.